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Research Article

Phytochemicals, Antioxidant and Anticancer Properties of *Camellia japonica* L. Mistletoe Extracts

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ABSTRACT

The present study aims to investigate the phytochemical profiles, *in vitro* antioxidant and antiproliferative properties of methanol and 70% ethanol extracts of *Camellia japonica* L. mistletoe. Both extracts were analyzed for contents of total flavonoid, total carotenoid and L-ascorbic acid, and antioxidant properties such as scavenging capacities (1,1-diphenyl-2-picrylhydrazyl, O_2^- and $NO^·$), ferrous ion chelating and reducing power. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to assess the antiproliferative properties against human cancer cell lines; MCF (human breast cancer cells), Hela (human cervical cancer cells), A375 (human malignant melanoma cells), HCT116 (human colon cancer cells), HepG2 (human liver cancer cells) and A549 (human non-small cell lung adenocarcinoma cells). The results showed that the methanol extracts of *Camellia japonica* L. mistletoe contained higher total flavonoids (16.237 g rutin equivalents/100 g) and carotenoids (49.175 g/100 g) with higher DPPH (SC_{50} = 0.6 mg/mL), superoxide (SC_{50} = 0.6 mg/mL), nitric oxide (SC_{50} = 0.5 mg/mL) radical scavenging and reducing power (IC_{50} = 1.1 mg/mL) activities than those of ethanol extracts ($p < 0.05$). In addition, methanol extracts showed much higher antiproliferative activity against human malignant melanoma A375 (IC_{50} = 118.1 µg/mL) and human colon cancer HCT116 (IC_{50} = 148.4 µg/mL) cells than ethanol extracts whereas higher inhibitory effects of human breast cancer MCF7 (IC_{50} = 139.9 µg/mL), human cervical cancer Hela (IC_{50} = 127.1 µg/mL) and human liver cancer HepG2 (IC_{50} = 84.2 µg/mL) cell proliferation in the ethanol extracts of *Camellia japonica* L. mistletoe. The results demonstrated the potential use of *Camellia japonica* L. mistletoe as a good antioxidant and anticancer effect.

Keywords: Anticancer activity, Antioxidant activity, Camellia mistletoe, Phytochemical compounds

Introduction

Mistletoes are a group of hemiparasitic plant growing on many host tree and shrub species. Thus, bioactivities of mistletoes could depend on its host plants where it grew [1]. Mistletoes have recently been described to be both agricultural pests and a threatened species in different parts of the world [2]. However, mistletoe is of great economic importance due to its major use in the medical treatment and management of many diseases for many years, both in traditional and complementary medicine [3, 4].

One of the mistletoe varieties that are often used in Korea as medicine is *Korthalsella japonica* [5]. It is known as *Camellia* mistletoe which is distributed in Jeju island, Korea. Recently there has been considerable interest in the antioxidant potential and antimicrobial activities of *Camellia* mistletoe extracts [6-9]. Although a lot of work was carried out with regard to the phytochemical and pharmacological evaluation of European mistletoe, *Viscum album* L. [10-13], but little is known of actual physiological benefits of *Camellia* mistletoe, *K. japonica*. Moreover, cytotoxic

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and anticancer activities of *Camellia* mistletoe are still rare and some reports would be useful to provide information on plants that
contain anticancer compounds [14, 15].

Today, discovery of antioxidant and anticancer agents from edible plants rich in polyphenols is of great interest [16]. Therefore, numerous efforts have been made to extract and separate various bioactive compounds from plant materials that can be in the food and pharmaceutical industries. As extraction solvent also plays a very important role in extraction of valuable bioactive compounds from the plant materials [6], it is necessary to determine the best solvent for extracting bioactive compounds from *Camellia* mistletoe.

The present study examined the effect of two organic solvents (methanol and ethanol) on the extraction yield and the content of flavonoids, carotenoids and L-ascorbic acids. The antioxidant and anticancer properties of the resulting extracts were also investigated.

Material and Methods

Plant materials and preparation of solvent extracts

Camellia mistletoe (*Korthalsella japonica* (Thunb.) Engl.) was collected during winter (February 2019) from Camellia Hill located in Andeok-myeon, Seogwipo-si, Jeju-do Province, South Korea and the voucher specimens (KHUP-0203) have been preserved in our laboratory for future reference. To prepare the solvent extracts of *Camellia* mistletoe, samples were freeze-dried. The ground powder of samples weas then extracted at room temperature twice by constant shaking for 72 h with solvents (1:10, w/v) of various polarities including methanol (100%) and ethanol (70%). The solutions were then filtered through Whatman paper and concentrated in vacuo using a rotary evaporator (Buchi Rotavapor R-200, New Castle, DE, US). The yield of solvent extracts was determined and residues were the taken to a laboratory for further analysis.

Determination of total flavonoids

Total flavonoid content was measured accordig to the precedure of Moon and Kim [7]. Briefly, an aliquot (15 μL) of each extract was mixed with 4.5 μL of 5% sodium nitrite, 60 μL of distilled water and 4.5 μL of 10% aluminum chloride. After incubation for 6 min, 60 μL of 1M sodium hydroxide solution (4%) was added to the mixture. Immediately, the mixture was made up to a final volume of 150 μL with distilled water and mixed thoroughly. After 15 min, the absorbance was determined at 510 nm versus a blank by using a Spectra MR microplate reader (Dynex Technologies, Inc., Chantilly, VA, US). Rutin which is a flavonoid glycoside was used as the standard.

Determination of total carotenoids

Determination of total carotenoids extract was performed according to previously developed method of Lima *et al*. [17]. The appropriate concentration (1-4 μg/mL) of *Camellia* mistletoe extracts were analyzed in a microplate reader at 470, 653 and 666 nm. The concentrations of carotenoids were determined according to the equations reported previously [18, 19].

Determination of ascorbic acid

Ascorbic acid determination was performed according to the method of Azard *et al*. [19] with slightly modified. The sample was extracted with 10 mL of 1% metaphosphoric acid for 45 min in the dark at room temperature and then filtered through Whatman paper. Plant extract (25 μL) was mixed with 225 μL of 2,6-dichloroindophenol and the absorbance was measured within 15 s at 515 nm against a blank solution. Content of ascorbic acid was calculated on the basis of the calibration curve of authentic L-ascorbic acid. The results were expressed as mg ascorbic acid per 100 g dry matter (mg AA/100 g).

DPPH, superoxide, and nitric oxide scavenging activities

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of the *Camellia* mistletoe extracts was measured according to the modified methods [7]. Briefly, freshly prepared 100 μL of 0.4 mM DPPH solution dissolved in methanol was added to the equal volume of each sample fraction. The reaction mixture was incubated for 10 min and the absorbance was measured at 517 nm using a Spectra MR microplate reader.

Table 1. Total flavonoid, carotenoid and L-ascorbic acid contents of Korean camellia mistletoe extracts

Solvent	Total flavonoid (mg/100 g)	Total carotenoid (mg/100 g)	L-Ascorbic acid (mg/100 g)
100% MeOH	16237±134 *	49175±849 *	543±78 *
70% EtOH	12705±114	16167±110	1258±101

Note: Each value is expressed as mean ± standard deviation (n = 3). *$p < 0.05$ compared to EtOH extract by Student's *t*-test

Table 2. IC_{50} value (mg/mL) in antioxidant activity of Korean camellia mistletoe extracts

Solvents	Radical scavenging			Ferrous ion chelating	Reducing power
	DPPH	Superoxide	Nitric oxide		
100% MeOH	0.6 ± 0.02 *	0.6 ± 0.01 *	0.5 ± 0.02 *	0.4 ± 0.01	1.1 ± 0.01*
70% EtOH	0.7 ± 0.01	0.7 ± 0.02	0.6 ± 0.01	0.3 ± 0.002	1.2 ± 0.02

Note: IC_{50} means the effective concentration at which the antioxidant activity was 50%, which was obtained by interpolation from linear regression analysis. Each value is expressed as mean ± standard deviation (n = 3). *$p < 0.05$ compared to EtOH extract by Student's *t*-test.
Need to report the results of the standard reference

The superoxide anion scavenging activity of *Camellia* mistletoe extracts was based on the method described previously [7]. The superoxide radicals were generated in a 50 mM sodium carbonate buffer (pH 10.5), 3 mM xanthine, 3 mM ethylenediamine tetraacetic acid (EDTA), 0.5 mM nitroblue tetrazolium (NBT) and 0.15% bovine serum albumin solution, and the solution of *Camellia* mistletoe extracts was then added. For the reaction, 0.25 units/mL of xanthine oxidase was added to the mixture, which was left standing at room temperature for 25 min. The absorbance was measured at 560 nm in a microplate reader.

Nitric oxide radical inhibition was measured by the Griess reduction [7]. Sodium nitroprusside in phosphate-buffered saline (10 mM, pH 7.0) was added to the extracts and the mixtures (100 µL) were incubated at 25°C for 3 h. Then, an equal volume of Greiss reagent was addded and kept for 5 min. The absorbance of these solutions was measured at 540 nm.

The results for scavenging activity of radicals, obtained from triplicate analyses, was expressed as SC_{50} values (mg/mL) which is the dose required to cause a 50% inhibition. Butylated hydroxytoluene (BHT) was used as positive control.

Ferrous ion chelating activity

The chelating ability was determined according to the method of Moon and Kim [7]. A volume of 5 µL of freshly prepared $FeCl_2$ (2 mM) was mixed with 250 µL of *Camellia* mistletoe extracts. A 10 µL of 5 mM ferrozine was added to the mixture and absorbance readings were taken after exactly 10 min at 25ºC.

Reducing power activity

The Fe^{3+} reducing power of *Camellia* mistletoe extracts was carried out as described previously developed method of Moon and Kim [7]. Different concentrations of the extract (200 µL, 0.125-2 mg/mL) were mixed with 200 µL of 200 mM phosphate buffer (pH 6.6) and $K_3Fe(CN)_6$ (200 µL, 1%). After incubation for 20 min at 50°C, 200 µL of 10% trichloroacetic acid solution was added to the mixture, and then centrifuged at 800 × g for 10 min. The upper layer of solution (100 µL) was mixed with deionized water (100 µL) and $FeCl_3$ solution (20 µL, 0.1%) prior to measuring the absorbance at 700 nm.

Cell culture

Anticancer activity of *Camellia* mistletoe extracts was determined against a panel of human cancer cell lines of MCF7 (breast), Hela (cervical), A375 (melanoma), HCT 116 (colon), HepG2 (liver), A549 (lung), besides one normal cell line (TK6). Cells were grown in DMEM (MCF7, Hela and A375), McCoy's 5A (HCT 116), MEM (HepG2), Ham's F-12 (A549) and RPMI 1640 (TK6) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 ºC.

Cytotoxicity assay

Cell viability was examined by the mitochondrial activity of the cells to cleave the tetrazolium salt the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Briefly, each extract was presolubilized in dimethylsulphoxide (DMSO) at 37°C to give a stock solution (1 mg/mL). Serial ten-fold dilutions were made from stock solution to give working concentrations of 0-200 µg/mL, making sure that the final concentration of DMSO in the tested dilutions was not higher than 1%. Confluent monolayers of cells were grown in 96-well culture plates for 24 h. Cells were incubated with various concentrations of the test extracts in triplicate at 37°C in a CO2 environment for 72 h. The negative control was performed using growth medium alone instead of plant extract. At the expiration of the 72 h treatment period, supernatants were removed from the wells and 25 µL of the MTT solution (2 mg/mL in PBS) was added to each well. The plates were incubated for 4 h at 37°C, and 125 µL of DMSO was added to each well to dissolve the formazan crystals. The optical density was determined at 540 nm using a Spectra MR microplate reader. The 50% inhibition of cell proliferation (IC50) was defined as the extract concentration required for the reduction of cell viability by half.

Statistical analysis

All data are presented as mean ± standard deviation of triplicate values. Significant differences between the groups were performed by using SPSS program (SPSS Inc. Chicago, IL, USA) using two-tailed Student's *t*-test. A *p*-value less than 0.05 was considered statistically significant.

Results and Discussion

Contents of total flavonoid, total carotenoid, and L-ascorbic acid

Phytochemicals such as flavonoids, carotenoids and ascorbic acid are known as biologically active compounds and responsible for antioxidant, antimicrobial, anticancer and antifungal activities [20, 21]. Flavonoids modulating lipid peroxidation involved in atherogenesis, thrombosis, and carcinogenesis. It has been proved that flavonoids pharmacological effects are correlating with their antioxidant potentialities [22]. Carotenoids and ascorbic acid are also important functional plant components [23-26]. Carotenoids, vegetable dyes present in the chloroplasts and chromophores, play an auxiliary role in the process of photosynthesis [23]. Vegetable carotenoids have antioxidant activity and can prevent vitamin A deficiencies [24]. Ascorbic acid is an antioxidant vitamin that acts synergistically with tocopherol to preserve antioxidant function in chronic disease states [25]. Ascorbic acid also has a protective function against photo oxidation processes [26]. However, polyphenols, carotenoids and ascorbic acid have not been adequately characterized in *Camellia* mistletoe, *K. japonica*. Therefore, we quantified these components in methanol and ethanol extracts of *Camellia* mistletoe (Table 1). Contents of total flavonoids and carotenoids were significantly higher in methanol extract (12705 and 16167 mg/100 g, respectively) ($p < 0.05$). However, ethanol extract of *Camellia* mistletoe contained higher L-ascorbic acid contents than methanol extract ($p < 0.05$) (Table 1). This result may be due to the type of solvents used for extraction and the solubility of secondary metabolites in mistletoes. Therefore, further experiments for identification of antioxidants in various extraction solvents are now in progress.

Antioxidant activities

Previous studies shown that the variability of the chemical composition and biological activity of mistletoe can also be associated with environmental conditions, i.e., ambient temperature, carbon dioxide concentration, pollution, soil fertility, and season [27-29]. Pietrzak and Nowak [29] reported that the chemical profile and biological activity of the plant material were closely related to the climatic conditions and location of the harvested plant. Higher levels of phenolic compounds and high antioxidant activity were found in extracts obtained from plant material collected in cold weather with the presence of snow and less sunshine [29]. Recently, we also shown that a considerable seasonal variation of inhibition of α-glucosidase, lipase, tyrosinase and NO˙ production was observed in *Camellia* mistletoe extracts, with February being the highest and May the lowest [6]. Therefore, samples harvested in February was employed to determine the bioactivities of *Camellia* mistletoe extracts.

Considering the high content of polyphenols, carotenoids and ascorbic acid compounds in *Camellia* mistletoe extracts (Table 1), we next evaluated their antioxidant activity by the free radical scavenging, chelating and reducing power methods. As shown in Table 2, methanol extracts of *Camellia* mistletoe exhibited significantly higher DPPH, superoxide and nitric oxide scavenging activities than ethanol extracts ($p < 0.05$).

BHT has been chosen as a positive control. Nearly all of the extracts showed a considerable free radical scavenging activity, although the activity was less than those of the positive controls at the same concentration (BHT scavenged 78.7% of the available free radicals, respectively, at the concentration of 100 μg/mL). These results suggest that *Camellia* mistletoe extracts are capable of avoiding free radicals' toxic effects.

However, methanol and ethanol extracts showed similar metal chelating ability (Table 2). The IC_{50} value of the chelating effect of methanol and ethanol extracts was 0.4 and 0.3 mg/mL, respectively (Table 2). Reducing power showed dose dependent increase in concentration absorption. ability of extract was increased with increasing concentration. IC_{50} values of methanol and ethanol extracts of *Camellia* mistletoe were 1.1 and 1.2 mg/mL, respectively (Table 2).

Effect of C. japonica mistletoe extracts on human cancer cell viability and cell growth

Recently, there is a greater global interest in nonsynthetic, natural medicines derived from natural sources due to better tolerance and minimum adverse drug reactions are compared to synthetic chemotherapeutic anticancer agents. Many cancer patients prefer herbal medicine because they are believed to be safe, cause less side effects and less likely to cause of dependency [30-32]. Thus, there is still a lot to be done in the search for novel natural chemopreventive compounds.

In the current research, the inhibitory activities of methanol and ethanol extracts of *Camellia* mistletoe on MCF7 (breast), Hela (cervical), A375 (melanoma), HCT 116 (colon), HepG2 (liver), A549 (lung) human cancer cell lines, and TK6 as a normal cell line were examined with the MTT assay. According to the results, both methanol and ethanol extracts of *Camellia* mistletoe showed antiproliferative effects against all tested human cancer cell lines, while there was no effect on the growth of TK6 as a normal cell, suggesting that the *Camellia* mistletoe extracts were selective towards cancer cells compared with those of normal. The data revealed that the increase in extracts concentration of up to 400 μg/mL could decrease the cell viability primarily in a concentration dependent mode in all cancer cell lines. The highest cytotoxic activity (IC_{50} of 84.2 μg/mL) was obtained against the HepG2 cell line by the ethanol extract of *Camellia* mistletoe (Table 3). 5-Fluorouracil (5-FU) was used as a positive control and exhibited a more pronounced cytotoxic effect against HepG2 cells with an IC_{50} of 1.91 μg/mL after 72 h of treatment. The IC_{50} of *Camellia* mistletoe-treated HepG2 was significantly lower than the IC_{50} of 5-FU after 72 h. The rank order of potency of the methanol and ethanol extracts of *Camellia* mistletoe for cell line was A549 (IC_{50} of 100.7 and 105.7 μg/mL), A375 (IC_{50} of 118.1 and 137.8 μg/mL), Hela (IC_{50} of 149.1 and 127.1 μg/mL), MCF7 (IC_{50} of 172.5 and 139.9 μg/mL) and HCT116 (IC_{50} of 148.4 and 213.2 μg/mL), respectively (Table 3).

Conclusion

Our results reveal that the *Camellia* mistletoe extracts have potent antioxidant and antiproliferative properties and are promising candidates to further investigate for functional uses. In addition, extraction solvents affected the amounts of flavonoids as well as antioxidant and antiproliferative properties of the *Camellia* mistletoe. Further molecular studies are undergoing to elucidate the mechanisms for the medical actions of *Camellia* mistletoe.

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